

Circadian Variation of Locomotor Ability Mediated by Nitric Oxide in the Cerebellum

A Senior Honors Thesis

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Abstract

Previous research has revealed circadian differences in locomotor ability corresponding to improved performance during active period. Nitric oxide (NO) production by the enzyme neuronal nitric oxide synthase (nNOS) exhibits circadian variation in the cerebellum. Considering the distribution and circadian variation of nNOS in the cerebellum, I hypothesized that NO could mediate daily rhythms in motor learning. The present study used the compound *7-nitroindazole* (7-NI) to selectively inhibit nNOS during only times of investigative interest to circumvent the possibility for developmental compensatory mechanisms in nNOS knock-out (nNOS^{-/-}) mice which lack nNOS throughout life. Mice were given intraperitoneal injections of either vehicle or 7-NI twice daily (1h prior to when lights were illuminated and before lights were extinguished) under a 12:12 (light:dark) photoperiod. Following lights on/off on the tenth day of injections groups were trained on an accelerating rotorod (4-40rpm) and tested 12 h later recording latency to fall. Four groups were formed based on treatment and time of behavioral analysis: A vehicle and a 7-NI group were trained in the morning and tested at night, and a vehicle and 7-NI group were trained at night and tested in the morning. I hypothesized 7-NI treatment would result in impaired locomotor ability and learning regardless of time of day and that vehicle treated groups would display the endogenous rhythm of locomotor ability. At the onset of the resting period vehicle treated mice exhibited impaired locomotor ability in accordance with the endogenous circadian rhythm, whereas 7-NI treated individuals lacked circadian variation of locomotor ability. NO expression was implicated in the circadian variation of locomotor ability. Immunocytochemical labeling of L-citrulline, a reliable marker of nNOS activity, will quantify the influence of nitric oxide expression in the cerebellum on circadian differences in locomotor ability and learning.

Introduction

The labile molecule nitric oxide (NO) was first characterized physiologically as an endothelium-derived vasodilator by its effect in relaxing smooth muscle cells in blood vessels (Palmer, R., et. al., 1987) and shortly after as a neurotransmitter in granule cells (Garthwaite et. al., 1988). NO is stoichiometrically produced as a free radical by the enzyme nitric oxide synthase (NOS) in the oxidation of L-arginine to L-citrulline. Three isoforms of NOS have been identified, neuronal (nNOS; (1)) in the brain, inducible (iNOS; (2)) in macrophages, and endothelial (eNOS;(3)) in blood vessels. NO exhibits a slight increase in polarity when compared to O₂, however retains the capacity to freely diffuse across cell membranes. Following production, NO diffuses from the point of origin, exhibiting a maximal 6 second half-life. Targets of NO include iron-containing heme groups, superoxide (O₂²⁻) anions, and most notably in the brain, soluble guanylyl cyclase (sGC) (Schuman, E.M., & Madison, D.V., 1994). NO production by constitutively expressed nNOS exhibits dependence on calmodulin, hence cytosolic Ca²⁺, thus NO is produced upon calcium influx to nNOS containing cells(Zhou, L. and Zhu, D., 2009). nNOS is most highly expressed in the cerebellum, however is expressed throughout the brain (Ayers, N.A., et al., 1996).

Cerebellar function influences balance, posture, coordination, and eye tracking as well as sequential, repetitive, and rapidly alternating movements. Inputs from the cerebral cortex, brainstem, and spinal cord encode the actual and intended movement of the organism to the cerebellum via mossy fibers. Climbing fibers convey motor errors perceived consciously by the cerebral cortex or by visual and/or vestibular systems. Purkinje cells differentially receive these inputs and provide inhibitory output to three pairs of deep cerebellar nuclei which fire in task-specific rates and patterns on cortical premotor and spinal cord motor systems. The function of the cerebellum is to compare the intended and actual movements of the organism and correct for discrepancies. Cerebellar lesion or removal does not affect strength or sensation, however, result in uncoordinated, spastic movement, hence the cerebellum modifies and coordinates the range, force, and timing of movements (Kandel, 2000).

The profoundly uniform organization of the cerebellar cortex was first documented by Ramón y Cajal in the early 1900s as having 5 types of neurons organized in 3 layers. Granule cells populate the innermost, granule cell layer of the cerebellar cortex interspersed with GABAergic Golgi interneurons. Granule cells axons project to the outermost molecular layer where they bifurcate to form parallel fibers. Parallel fibers form excitatory synapses on inhibitory basket, stellate, and Purkinje cells within the molecular layer. The cell bodies of Purkinje cells form the intermediate, Purkinje cell layer of the cerebellar cortex. Inhibitory Purkinje cells provide the sole, inhibitory output of the cerebellar cortex to deep cerebellar nuclei which is differentially influenced by mossy and climbing fiber input (Reviewed in Ito, 2001; Ito, 2006; Kandel, 2000).

The cerebellum contributes to the improvement seen in the range, force, and timing of repeated movements by modifying the relative efficacy with which excitatory mossy and climbing fibers stimulate Purkinje cell output on deep cerebellar nuclei. Long-term potentiation (LTP) at the parallel fiber-Purkinje cell synapse is the increase in synaptic efficacy seen following parallel fiber input to Purkinje cells in the absence of perceived error. If an error is perceived, climbing fiber input from the sensory field of the error induces long-term depression (LTD), a decrease in efficacy, at the parallel fiber-Purkinje cell synapse. LTD temporarily "relieves" the Purkinje cell of parallel fiber input, hence "relieves" deep cerebellar nuclei from its inhibition - altering their firing pattern on the respective motor system. It is through this "error-driven" modification of rate and pattern of deep cerebellar nuclei discharge on motor systems as mediated by Purkinje cell synaptic efficacy that the cerebellum contributes to the improvement in the range in force of motor tasks seen with repeated trials. The nitric oxide pathway is 1 of 7 routes of inducing cerebellar LTD (Ito, 2001)

In the cerebellum nNOS is found in granule, basket, and stellate cells. Following stimulation from mossy fiber inputs, which induces LTP at parallel fiber-Purkinje cell synapses, NO is produced in parallel fibers which simultaneously induces LTD at the parallel fiber-Purkinje cell synapse. During conjunctive mossy and climbing fiber input, glutamate spillover at climbing fiber-Purkinje cell synapses spurs NO production in parallel fibers, basket and stellate cells, shifting the biochemical cascade equilibria to LTD

in neighboring cells (reviewed in Ito, 2001). In this light NO in the cerebellum functions to coordinate the activity of neighboring cells.

Wild-type mice exhibit a circadian rhythm of locomotor activity corresponding to comparatively improved locomotor performance during the active period (Kriegsfeld, L.J. et al. 1999a). Circadian variation of NOS activity has been demonstrated throughout the brain, with peak NO production observed during the active period in all regions (Ayers, N.A., et al., 1996). Additional evidence has been provided which suggests that NOS exhibits peak activity in the molecular layer of the cerebellar cortex during the resting period (Demas, G. E., unpublished results). Because the Ayers study assessed NO production in homogenized tissue and the Demas study in cerebellar slices, these findings may not necessarily conflict. As opposed to non-specific NOS inhibitor-treated mice, the endogenous circadian locomotor activity rhythm and photoperiod entrainment capacity are not disrupted in nNOS genetic knock-out (nNOS^{-/-}) mice (Kriegsfeld, L.J. et al. 1999a), but nNOS^{-/-} mice fail to exhibit the expected improvement in locomotor ability during the active period seen in WT mice as assessed by balance on a pole (Kriegsfeld, L.J. et al. 1999b). However, a number of considerations must be made when considering such data with regards to the absence of the gene throughout development, an example of which being the potential for compensatory mechanisms which may serve to spare function.

To circumvent confounding influences posed by compensatory mechanisms in the knock-out model and non-specific inhibition of NOS seen in traditional NOS inhibitors, the present study uses a 7-*nitroindazole* (7-NI) to selectively inhibit nNOS. 7-NI is reported to reversibly bind with nNOS in an L-arginine fashion with equal potency to non-selective NOS inhibitors, preventing binding and subsequent NO production (Babbedge, R.C., et al., 1993). The specificity of 7-NI for nNOS and not eNOS has been ascribed to its differential uptake by neurons and epithelial cells (Southan & Scabó, 1996). The present study aims to selectively inhibit NO production in neuronal populations during only times of investigative interest.

I examined the influence of NO on circadian variation previously observed in locomotor ability. I hypothesized that NO may mediate the circadian differences observed in motor ability such that when

nNOS production of NO is blocked, circadian variation of locomotor ability is likewise impaired. Conversely, in animals where NO production is undisturbed circadian variation of locomotor ability will be observed corresponding to improved locomotor ability during the active period. In accordance with the role of NO in cerebellar LTD, selective inhibition of NO production in neuronal populations should elicit impaired motor task learning corresponding to reduced latencies to fall in testing trials. Quantification of L-citrulline production in cerebellar neurons will indicate a positive correlation between rotorod ability and learning and NO production in the cerebellum. Lack of NO production in the cerebellum will result in resting period-like rotorod ability with reduced learning.

Methods

Three separate cohorts of mice were of mice were analyzed using the following protocol. Although procedural amendments were made and will be noted for each successive cohort, the majority of the procedure remained constant for all cohorts.

Animals

A total of 116, six-week-old male C57bl/6 mice were obtained from Jackson Laboratory. Upon arrival mice were singly housed in polypropylene cages (dimensions: 27.8 x 17.5 x 13 cm) and provided food (Harlan Tech. Lab Rodent Diet 8640) and filtered tap water *ad libitum*. Mice were housed under a 12:12 (light:dark) photoperiod for the duration of the experiment with the light phase beginning at 0600h Eastern Standard Time (EST) in the first cohort, and 0700h EST in the second and third cohorts.

Treatment

Groups were randomly formed with regards to time of testing and treatment administered. Following a one-week photoperiod habituation period, mice were weighed to calculate treatment dosages which would be administered at 50 mg/kg. 7-NI solution was prepared twice daily in corn oil at 12.5

mg/mL. For the first and second cohorts, 7-NI solution was left on a shaker in the dark no fewer than 10h prior to use, whereas in the third cohort 7-NI was sonicated until in solution, then incubated at 37°C for no fewer than 10h in the dark prior to use. Intraperitoneal (i.p.) injections were administered twice daily beginning at 0500h and 1700h in the first cohort, and 0600h and 1800h in the second and third cohorts for 10 days prior to behavioral testing and were continued throughout testing. Mice were placed back in their cages following injections.

Behavioral Testing

In all cohorts four training trials and three testing trials - a trial consisted of one run on the accelerating rotorod from 4-40 rpm - were recorded for each individual on the accelerating rotorod. In the first two cohorts behavioral testing was conducted within the vivarium, whereas in the third cohort mice were transported via a light-tight box to a behavioral suite following injections. Trials began 1h after injections (0600h and 1800h for the first cohort and 0700h and 1900h for the second and third cohorts) to allow adequate time for 7-NI absorption. In the first cohort no maximum time limit was enforced, whereas in the second and third cohorts trials were terminated after 300 s. For the first and second cohorts, following each trial mice were placed back into their cages on the cage rack while other mice were tested. After all mice had completed the round of trials, the next round of trials would be conducted using the same order of mice. Sequential trials were used in the third cohort of mice. Following each trial, mice were placed back in the cage on the testing table for 10 s before the next trial would commence. Ethanolic solution (70%) was used to sterilize the rotorod between trials in the first and second cohorts, and after sequential trials for each individual in the third cohort.

Perfusions

Following the final testing trial, mice were anesthetized with isoflurane vapors and given an overdose of sodium pentobarbital. Blood samples were collected from the retro-orbital sinus and placed

on ice. Perfusion of ice cold phosphate buffered saline (PBS) through the left ventricle of the heart was followed by a fixative solution (3% paraformaldehyde/1% glutaraldehyde in PBS). Brains were removed and postfixed for no longer than 8h and then transferred to 30% sucrose solution. Upon sinking in the sucrose solution, brains were flash frozen using dry ice and stored at -80°C until processing.

Immunohistochemistry

One brain from randomly selected mice in each group in the first cohort were coronally sectioned (40 µm) on a cryostat at -23°C. Sections were collected in 30% sucrose antifreeze solution and stored at -20°C until immunohistochemical processing. Free-floating sections were washed and permeabilized 3 x 5 min with 0.5% Triton-X (TX) 0.1M phosphate buffered saline (PBS) solution then incubated for 1 h on a shaker in 0.5% sodium borohydride and 0.2% sodium metabisulfite PBS solution. Following incubation, sections were washed again using similar protocol, then incubated for an additional 1 h in 5% normal goat serum (NGS) and 0.5% TX in 0.1 M PBS solution on a shaker. The NGS solution was removed and 20 µL (1:1000) rabbit anti-citrulline antibody in 5% NGS, 0.5% TX, 0.1M PBS solution was applied and left to incubate for 48 h on a shaker. The sections were washed again using the said protocol, then 20 µL (1:1000) biotinylated goat anti-rabbit antibody in 0.1M PBS with 0.5% TX and 5% NGS was applied and left to incubate for 90 min on a shaker. During the incubation time, avidin-biotin complex (ABC) was prepared and left to sit for 30-60 min at -4°C. Sections were washed 3 x 5 min with 0.1 M PBS, then ABC was applied to amplify the secondary, biotinylated goat anti-rabbit, antibody and left to incubate for 1 h on a shaker. Following another wash, 3 x 5 min with 0.1 M PBS, L-citrulline staining was developed using diaminobenzidine (DAB) with hydrodogen peroxide and nickel for 45 s. After a final wash, 2 x 5 min in 0.1 M PBS, sections were mounted on slides with distilled water, dehydrated for 2 min each in 95%, then 3 x 100% enthanolic solution, cleared with 3 x 2 min washes in xylene, then coverslipped using Permount.

Behavioral Data Analysis

Latency to fall and fecal boli data were analyzed using SPSS. Uni-, and multivariate ANOVA and Student's T-test comparisons of locomotor performance as assessed by latency to fall, time of day, and treatment were made between groups. Findings were considered significant if ($p \leq 0.05$).

Results

I examined the circadian locomotor influence of nNOS inhibition by 7-NI with the accelerating rotarod. Because the experimental protocol was modified between 3 cohorts of mice, the data from each cohort must be interpreted separately. In fact, a multivariate ANOVA indicates significant differences between cohorts ($p < 0.05$).

In the first cohort a multivariate ANOVA comparing time of testing and treatment revealed a trend of treatment on the latency to fall in training trials but not in testing trials. A Univariate ANOVA follow-up analysis confirmed this treatment effect in training trials ($F_{(1,1)} = 6.329$, $p < 0.05$; Figure 1, left).

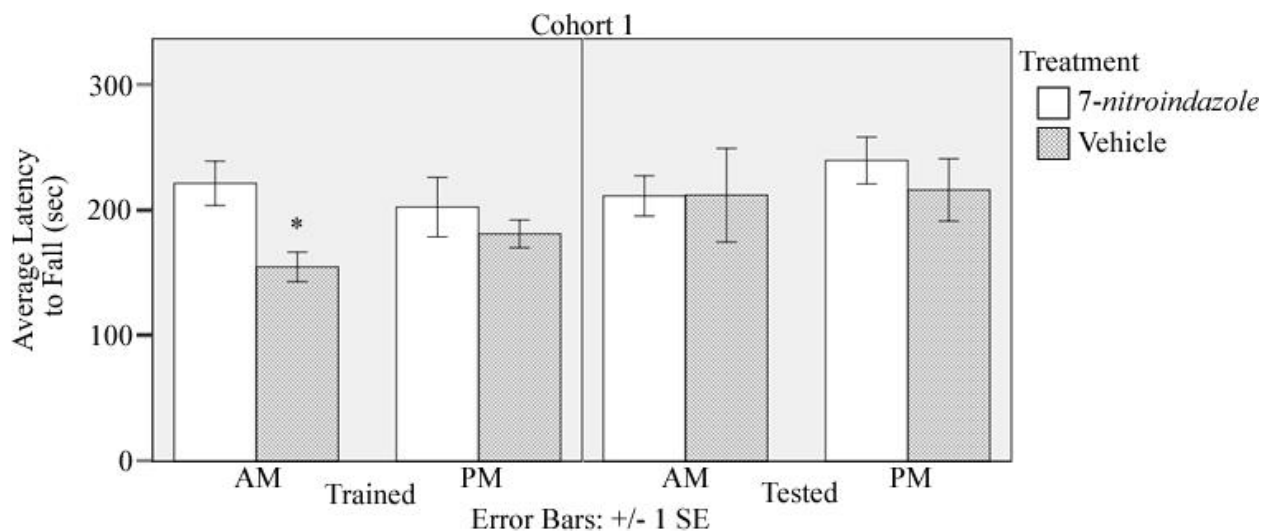


Figure 1. Histogram showing average latency to fall in training and testing trials sorted by time of day and treatment for animals in the first cohort (N = 28). A significant effect of treatment on average latency to fall in AM training trials was observed ($F_{(1,1)} = 6.329$, $p < 0.05$)

There was no effect of 7-NI treatment in the second cohort of mice in training or in testing, presumably due to 7-NI precipitating out of solution before injections (Figure 2, below).

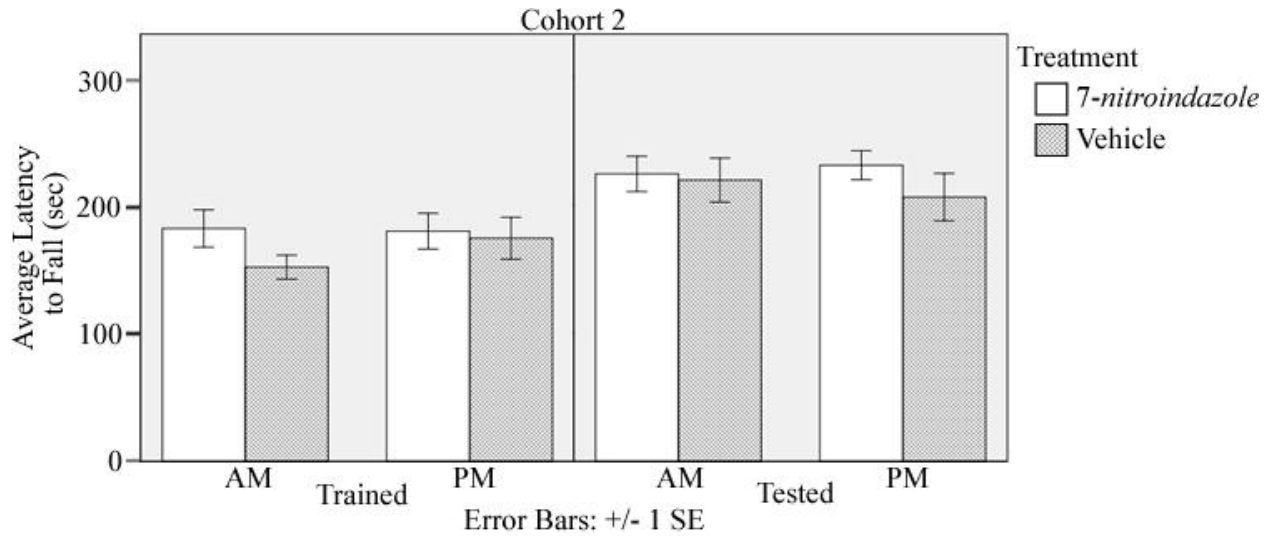


Figure 2. Histogram showing average latency to fall in training and testing trials sorted by time of day and treatment for animals in the second cohort (N = 32). No effect of treatment on average latency to fall in training or testing trails was observed.

In the third cohort an effect of treatment was reestablished in training, indicated by a multivariate ANOVA ($F_{(1,1)} = 3.549, p = 0.047$), however in the opposite direction. Univariate ANOVA follow-up analysis confirms this finding ($F_{(1,1)} = 7.371, p < 0.05$; Figure 3, left). As in all cohorts there was no effect of treatment on testing trials.

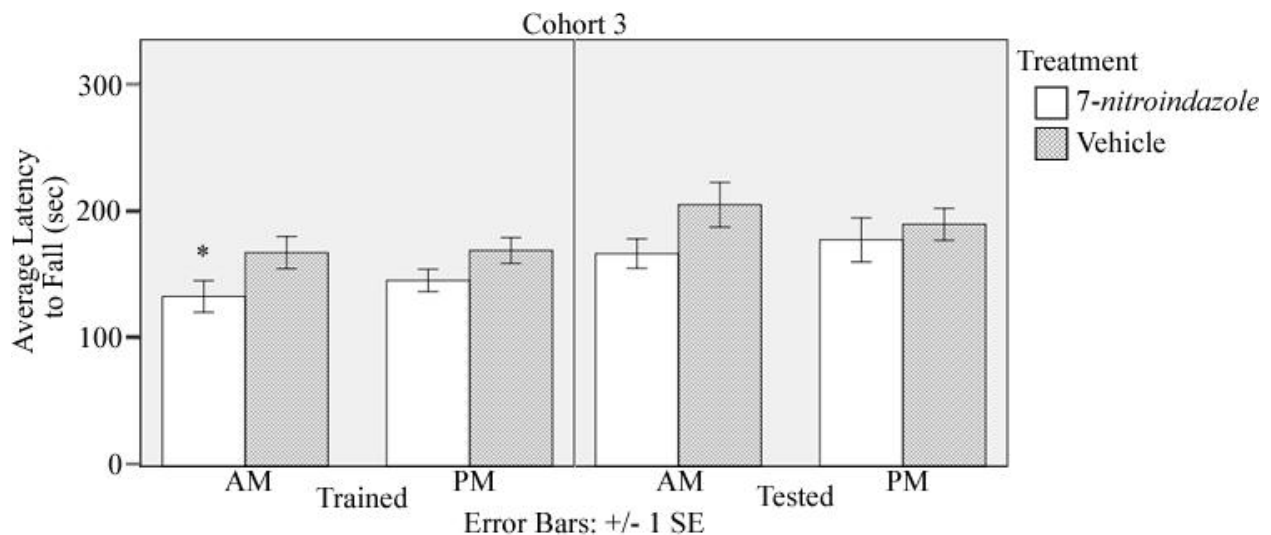


Figure 3. Histogram showing average latency to fall in training and testing trials sorted by time of day and treatment for animals in the third cohort (trained: N = 56, tested: N = 26). An effect of treatment on average latency to fall in AM training trails was observed ($F_{(1,1)} = 7.371, p < 0.05$)

There were no differences observed between treatment, or time of day groups in motor learning as assessed by the difference in average testing and training latencies to fall. Fecal boli production, a measure of sympathetic activation (Taché, Y., & Bonaz, B., 2007), was significantly different with regards to time of day in all cohorts as shown by a Student's T-test in both training ($F_{(1,1)} = 21.913, p < 0.001$) and testing ($F_{(1,1)} = 4.583, p < 0.001$). A significant effect of treatment on fecal boli production in training trials was identified by a Student's T-test in the first ($F_{(1,1)} = 0.026, p < 0.05$) and third cohorts ($F_{(1,1)} = 1.957, p < 0.001$), but not in the second cohort. This effect was also seen in testing of the third cohort ($F_{(1,1)} = 6.306, p < 0.01$), but not in the first two cohorts.

Immunohistochemical staining for L-citrulline has identified nNOS activity during the active period (below). Sections shown were taken from the first cohort of mice to verify 7-NI treatment. As noted, there was a significant effect of treatment in the first and third cohorts.

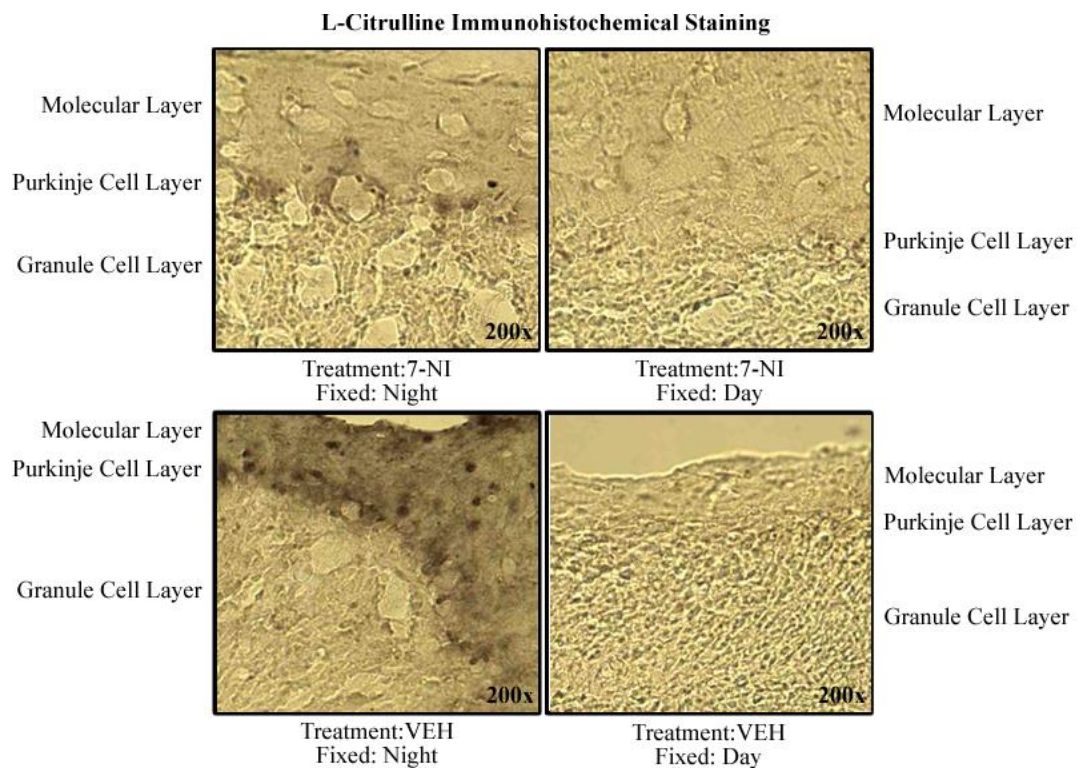


Figure 4. Quantification of nNOS activity through L-citrulline staining of cerebellar slices (40 μ m) viewed at 200x magnification from 7-nitroindazole and vehicle-treated mice fixed at the onset of day and night. L-citrulline visualization indicates NO production occurs during the active phase (bottom left). Conversely, nNOS appears to be silent during the resting period (right). 7-NI treatment greatly reduced NO expression during the active period (night; top left).

Discussion

Although a significant effect of 7-NI on locomotor ability was observed, lack of significant circadian variation of locomotor ability in vehicle-treated groups limits the statistical power of these data. The present findings cannot be attributed to inhibition of nNOS exclusively in the cerebellum as 7-NI was administered intraperitoneally. Indeed, 60 minutes after i.p injections of 7-NI nNOS was shown to be significantly inhibited in the cerebellum, cerebral cortex, hippocampus, and adrenal gland (Babbedge, R.C. et al., 1993). With regards to the lack of circadian variation in vehicle treated animals, I suspect twice daily intraperitoneal injections may have interfered with the circadian rhythms of individuals in all cohorts, whether it be due to the injections, handling, sore abdomens, or large pockets of oil reported in the animals *post mortem*. It must also be noted that corticotropin-releasing hormone (CRH) release from climbing fibers is also implicated in LTD induction at Purkinje cell synapses (Miyata, M. et al., 1999) and that this system may also have been disrupted due to the treatment regimen. Immunohistochemical staining for L-citrulline, a reliable marker of nNOS activity (Martinelli, G.P.T., et al., 2002), has identified NO to be produced during the active period (night) in the cerebellum, however, as circadian variations appear to have been disrupted, these data must be interpreted with caution. The present data show that NO expression in the cerebellum is without effect on the circadian variation of motor learning, however the methods employed did not permit a complete examination of the role of NO in this behavior.

In behavioral tests inhibition of nNOS by 7-NI has been shown to be antinociceptive (Moore, P.K., et al., 1993) and contribute to aggression (Demas, G.E., et al., 1997). Inhibition of nNOS has also been shown to promote the release of norepinephrine (Kiss, J.P. et al., 1996) and dopamine from hippocampal and striatal neurons (Silva, M.T. et al., 1995) as well as modulate serotonin turnover (Chiavegatto, S., et al., 2001). At low doses (1-5 nmol) 7-NI had anxiolytic effects in the elevated plus maze and antidepressant effects in the forced swim test, whereas at high doses (10 nmol) the opposite effects were observed (Spiacchi, Jr., A., et al., 2008). In the first two cohorts, 7-NI was noted to have begun precipitating out of solution before injections. In the third cohort of mice, sonication and

incubation of 7-NI (Chi, Z. et al., 2003) aided in keeping 7-NI in solution for injections. Indeed, the largest effect of treatment was observed in the third cohort. If the dose in the third cohort was above the threshold for anxiogenic and depressogenic effects of 7-NI, and the first two cohorts were below, it may help to explain the reversal of trends observed. This may also have implications regarding the CRH-mediated LTD induction.

Stress-elicited responses have long been documented in rodents (Selye, H., 1937). In response to environmental stressors, CRH is released by the hypothalamus signals the release of norepinephrine (NE) from the locus coeruleus (Valentino, R.J. et al., 1993) and adrenocorticotrophic hormone (ACTH) from the pituitary, which further signals the release of glucocorticoids (GCs) and catecholamines (NE, and epinephrine (EPI)) from the adrenal glands. CRH immunoreactive cells are located throughout the central nervous system (Cummings, S. et al., 1983). Exogenous CRH administration has been shown to increased heart rate, blood pressure, activation in areas of the brain associated with arousal (Ehlers, C.L. et al., 1983) and in behavioral tests be anxiogenic (Fisher, L.A., 1989), enhance learning at low doses, and stimulate locomotor and exploratory activity (Sutton, R.E. et al., 1982; Eaves, M. et al., 1985). In the cerebellum, immunohistochemistry localizes CRH to climbing fibers (Palkovits, M. et al. 1987) and *in situ* hybridization of the CRH receptor mRNA identifies type 1 CRH receptors in deep cerebellar nuclei as well as in Purkinje and granule cells (Potter, E. et al., 1994). Climbing fiber-released CRH has been implicated in LTD at Parallel fiber-Purkinje cell synapses in the cerebellum, indicated by the ability of CRH receptor antagonists to abolish LTD induction, but not maintenance, in cultured Purkinje cells. This study further showed that the CRH-mediated response is without effect on the NO-mediated response (Miyata, M. et al., 1999). Taken together, the anxiogenic/depressogenic effects of high doses of 7-NI coupled with increases in CRH due to treatment stress may help to explain the reversal of trends seen in the third cohort.

Locomotor ability and learning in third cohort mice were assessed using a different rotarod protocol than in the first two cohorts. In the third cohort, only 10 s was allotted between trials, whereas in

the first and second cohorts, an average of 96, and 26 min between training trials and 81, and 19 min between testing trials, respectively, were given. It has been experimentally demonstrated that LTD progressively develops over an hour following induction (Karachot, L. et al., 2001). Additionally, there were notable age differences between cohorts. In the first and second cohorts, mice were tested between 8-12 weeks of age, when nNOS expression is high in the cerebellum. In the third cohort, mice were tested at 23 weeks of age. Age-dependent decrease in nNOS expression in the cerebellum has been experimentally documented in rats, indicating a significant decrease between 2 and 6 months of age (Yu, W.-J. et al., 2000). If circadian variation of NO expression in the cerebellum does mediate circadian variation of locomotor ability, speculated age-dependent decreases of nNOS in the third cohort of mice may aid in explaining the observed lack of circadian variation.

Procedural changes implemented after the first and second cohorts warrant caution when considering the overall data. As noted in the methods section, I attribute the lack of treatment effect seen in the second cohort to 7-NI precipitating out of solution. I propose the reverse of trends seen in the third cohort may be due to the lack of appropriate time for LTD induction between trials and/or side effects of 7-NI associated with inhibition of nNOS coupled with treatment stress. In all cohorts, circadian variation of locomotor ability was not observed in vehicle or 7-NI treated animals. No conclusions can be made from the present data. Systemic inhibition of nNOS has shown to confound the ability to test any specific function of NO as evidenced by the array of effects seen in knock-out and pharmacological studies. Future studies may seek methods of administering nNOS inhibiting compounds to regions of interest *in vivo* while minimizing disturbance to the subjects.

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Through conducting an honors thesis here at The Ohio State University it is obvious to me that I have learned a lot and even more obvious that there is a lot more to learn. I would like to thank Dr. Randy Nelson for providing me the opportunity, Dr. Joanna L. Workman for preliminary assistance, and James Walton for all the help and especially for knowing when not to intervene on a learning experience.

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